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Remarks

In the Office Action dated November 7, 2004, claims 53, 57-58 and 60-103, in the above-identified U.S. patent application were rejected. Reconsideration of the rejections is respectfully requested in view of the above amendments and the following remarks. Claims 53, 57-58, 60-63 and 65-103, remain in this application, claims 1-52, 54-56 and 64 have been canceled.

Claims 53, 57-58 and 60-103 were rejected under 35 USC §112, first paragraph, as lacking enablement for the detection of antibiotic resistance in any microorganism by detecting any mutation in any peptidyltransferase center of 23S rRNA. Applicants respectfully point out that the problem underlying the present invention is not the identification of new point mutations in the peptidyl transferase center responsible for antibiotic resistance. Instead, the present invention exploits known point mutations responsible for antibiotic resistance in a diagnostic test, as indicated on page 5, lines 32 –38 of the present specification. Macrolide resistance is mediated by specific positions which have a conserved nature in a very wide variety of transferase centers of microorganisms. A person skilled in the art would take these conserved positions, which are given on page 17, line 18 of the description, into consideration when looking for point mutations responsible for antibiotic resistance. Prior to the present invention, no other point mutation was detected in this region which was responsible for antibiotic resistance. A person skilled in the art would therefore, regard the other positions of the peptidyl transferase center as not being relevant regarding antibiotic resistance and the selection of a point mutation as a matter of routine

experimentation. Attached is a paper by Wang et al. which indicates that the peptidyl transferase center consists of a limited and defined number of nucleotides (see Figure 3).

As pointed out on page 3, line 23 to 26, of the present application, macrolide antibiotics act by blocking the peptidyl transferase center. Macrolide antibiotics act by one of the point mutations 2057, 2058, 2059 or 2611 given in the present description (see the enclosed papers Wang et al. or Occhialini et al. which refer to the relevant state of the art, in particular Wang et al., Figure 3, p. 1956, left-handed column). Thus, applicants contend that a common macrolide mode of action enables the invention for all macrolide antibiotics even if it is exemplified only by clarithromycin. Applicants also contend that since evidence for a common macrolide mode of action is presented, a person skilled in the art need not assign specific point mutations in the peptidyl transferase center to specific macrolide antibiotics as stated in the sentence bridging pages 4 and 5 in the pending Office Action.

The office action also indicates that the generalization to any microorganism based upon the example of *H. pylori* is not enabled. As demonstrated by Table 7 of the present specification, the peptidyl transferase center, in particular the region covering positions 2057, 2058 and 2059, is strongly conserved among a large variety of pathogenic microorganisms, including gram-negative (e.g. *Helicobacter*) and gram-positive (e.g. *Mycobacterium*) species. A person skilled in the art would conclude that, due to the common mode of action of macrolide antibiotics, and due to the large

degree of homology of the peptidyl transferase center in bacteria species, point mutations in different species leading to antibiotic resistance would occur at identical positions. Thus, applicants contend that the detection of antibiotic resistances by detection of point mutations in the peptidyl transferase center is enabled in any bacterial species by the *Helicobacter* example of the present invention. In view of the above discussion, applicants request that the enablement rejections be withdrawn.

Claims 53, and 57-91 were rejected under 35 USC § 112, second paragraph, as indefinite. The claims have been amended deleting the language found indefinite. In view of these amendments applicants request that this rejection be withdrawn.

Claims 53, 57-58, 60-85, 92, 93 and 101-103 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Amann 1995 and Amann 1990. As discussed in prior responses, Versalovic did not successfully discriminate point mutations using *in situ* hybridization. The Amann references do not cure this deficiency as Amann (1990) is only relevant for single-mismatch discrimination. On page 765, Amann (1990) points out that it was possible to distinguish between *Fibrobacter* strains that differed by a single-mismatch in 16S rRNA (strains S85 and HM2, right-hand column). However, the ability to discriminate complementary- from single-mismatch hybrids varied markedly between different probe and target sequences. The authors concluded that single-mismatch discrimination must be empirically established. It is important to note that Amann et al. (1990) performed *in situ* hybridization with cells which

were harvested in the mid-log phase (when cells are growing fastest) in order to optimize the rRNA content (page 762, right-hand column, "Culture Conditions"). This is an important technical feature of the Amann procedure since **Amann et al. (1995) point out that slowly growing cells are difficult to detect because of their low cellular RNA content** (see page 159, left-hand column). As can be seen from Amann (1990), page 767, left-hand column, and Table 2, even under optimal conditions provided by cells growing in the mid-log phase, there is a weak discrimination between the *Fibrobacter* strains LH1 and JG1 on the one hand, and the strains NR9, DR7 and C1a on the other hand, differing in a single position in the 16S rRNA region of interest.

Amann's data (1990) regarding *in situ* hybridization of bacteria in fresh specimens demonstrate that the *Fibrobacter intestinales* strain NR9 grown under optimal conditions, can be detected by the specific probe "intestinales 1+2", when the cells are added to the fresh specimen (page 770, left-hand column). In this context, Amann (1990) demonstrates that this specific probe also hybridizes to a fraction of cells from the fresh specimen. However, since these cells are not further characterized, no conclusion can be drawn as to the nature of these cells. In any event, no single-mismatch-discrimination was shown for fresh specimens.

Amann (1990) could be interpreted as teaching that it is possible to perform single-mismatch discrimination by *in situ* hybridization under optimal conditions. However, Amann indicates that it is necessary to employ cells growing in the mid-log phase which thus have an optimal rRNA content. In view of this, a person skilled in the art would not have a reasonable expectation of

success when considering the use of cells growing under suboptimal conditions, particularly cells obtained from biological specimens kept in a presumptive medium which is designed to keep cells alive but which suppresses growth of the organisms to a large extent (see page 19 of the present description). In general, microorganisms grow rather slowly in most environmental conditions.

Applicants contend that the combination of the teachings of Versalovic with Amann (1990) and Amann (1995), would lead a person skilled in the art to arrive at a method for single-mismatch discrimination suitable for cells which were kept in culture in order to optimize rRNA content before the hybridization is performed. Due to the lack of reasonable expectation of success, a person skilled in the art would not arrive at a method for single-mismatch discrimination by hybridization in biological specimens without prior cultivation. Claim 53 has been amended to clarify that the microorganisms are not cultured prior to contact with the hybridization probe. This limitation was previously recited in claim 64 and thus does not raise any new issues which would require a further search.

Claims 86-90 and 94-97 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Amann (1995) and Amann (1990) in view of the Stratagene catalog. As discussed above, the combination of Versalovic, Amann (1990) and Amann (1995) does not suggest or disclose a method for single-mismatch discrimination by hybridization in biological specimens without prior cultivation. The Stratagene catalog does not cure this deficiency as Stratagene was cited only for the disclosure of a kit and does not suggest a method for single-mismatch discrimination by hybridization. In view of the above

discussion regarding the rejection over Versalovic in view of Amann (1995) and Amann (1990), applicants request that this rejection be withdrawn.

Claims 91 and 98-100 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Amann (1995) and Amann (1990) in view of the Stratagene catalog further in view of Morotomi. As discussed above, the combination of Versalovic, Amann (1995), Amann (1990) and the Stratagene catalog does not suggest or disclose a method for single-mismatch discrimination by hybridization in biological specimens without prior cultivation. Morotomi does not cure this deficiency as Morotomi was cited only for the disclosure that *H. pylori* can be detected using a urease indicator. In view of the fact that Morotomi in combination with Versalovic, Amann (1995), Amann (1990) and the Stratagene catalog does not suggest or disclose a method for single-mismatch discrimination by hybridization in biological specimens without prior cultivation, applicants request that this rejection be withdrawn.

Claims 92, 93, 101 and 102 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Hiratsuka and Gingeras. Hiratsuka was cited for the disclosure of the complete sequence of the 23S rRNA of *H. pylori*. Gingeras is directed to oligonucleotide sequences based on the *M. tuberculosis* *rpoB* gene. Gingeras relates to hybridization on micro arrays which is not suitable for *in situ* hybridisation and even the assay conditions for micro array hybridization cannot be applied to *in situ* hybridisation. Gingeras' techniques require isolation of nucleic acids, for instance by PCR and its labeling (see Gingeras, column 8). Although Gingeras was cited for the disclosure of a

method for making probes which can distinguish between wildtype and mutant nucleic acids that differ by one nucleotide, col. 9, lines 57-65, indicates that **differentiation between species based on one different nucleotide is not usually possible** and thus hybridization patterns from multiple regions are usually observed. The detection of an increasing number of these differences allows one to classify the organism. Column 10, lines 10-12 indicates that when a single probe set is used it will not usually be able to define the differences in sequence between the target and the reference sequence. Thus, Gingeras does not teach or disclose that a single probe set can be made which can distinguish between wildtype and mutant nucleic acids that differ by one nucleotide. As discussed above, Versalovic did not successfully discriminate point mutations using *in situ* hybridization. Thus, one skilled in the art would not be motivated make the presently claimed oligonucleotides in view of the combination of Versalovic, Hiratsuka and Gingeras.

In addition, none of the cited references teaches the specific oligonucleotides claimed in claim 92. These oligonucleotides are significantly smaller than the sequence disclosed in Hiratsuka and there is no guidance as to what part of Hiratsuka's sequence is important.

Claims 86-90 and 94-97 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Hiratsuka and Gingeras further in view of the Stratagene catalog. As discussed above, the combination of Versalovic, Hiratsuka and Gingeras does not suggest or disclose the claimed oligonucleotides. The Stratagene catalog does not cure this deficiency as

Stratagene was cited only for the disclosure of a kit and does not suggest a method for single-mismatch discrimination by hybridization or kits containing oligonucleotides useful for determining macrolide antibiotic resistance in microorganisms by in-situ hybridization. In view of the above discussion, applicants request that this rejection be withdrawn.

Claims 91 and 98-100 were rejected under 35 USC §103(a) as unpatentable over Versalovic in view of Hiratsuka, Gingeras, and the Stratagene catalog further in view of Morotomi. As discussed above, the combination of Versalovic, Hiratsuka, Gingeras and the Stratagene catalog does not suggest or disclose the claimed oligonucleotides or kits. Morotomi does not cure this deficiency as Morotomi was cited only for the disclosure that *H. pylori* can be detected using a urease indicator. In view of the fact that Morotomi in combination with Versalovic, Hiratsuka, Gingeras and the Stratagene catalog does not suggest or disclose a kit for determining macrolide antibiotic resistance in microorganisms by in-situ hybridization, applicants request that this rejection be withdrawn.

Applicants respectfully submit that all of claims 53, 57-63 and 65-103 are now in condition for allowance. If it is believed that the application is not in condition for allowance, it is respectfully requested that the undersigned attorney be contacted at the telephone number below.

In the event this paper is not considered to be timely filed, the Applicant respectfully petitions for an appropriate extension of time. Any fee for such an

extension together with any additional fees that may be due with respect to this paper, may be charged to Counsel's Deposit Account No. 02-2135.

Respectfully submitted,

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Site-Specific Mutations in the 23S rRNA Gene of *Helicobacter pylori* Confer Two Types of Resistance to Macrolide-Lincosamide-Streptogramin B Antibiotics

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Clarithromycin resistance in *Helicobacter pylori* is mainly due to A-to-G mutations within the peptidyltransferase region of the 23S rRNA. In the present study, cross-resistance to macrolide, lincosamide, and streptogramin B (MLS) antibiotics (MLS phenotypes) has been investigated for several clinical isolates of *H. pylori*. Two major types of MLS resistance were identified and correlated with specific point mutations in the 23S rRNA gene. The A2142G mutation was linked with high-level cross-resistance to all MLS antibiotics (type I), and the A2143G mutation gave rise to an intermediate level of resistance to clarithromycin and chlindamycin but no resistance to streptogramin B (type II). In addition, streptogramin A and streptogramin B were demonstrated to have a synergistic effect on both MLS-sensitive and MLS-resistant *H. pylori* strains. To further understand the mechanism of MLS resistance in *H. pylori*, we performed in vitro site-directed mutagenesis (substitution of G, C, or T for A at either position 2142 or 2143 of the 23S rRNA gene). The site-directed point mutations were introduced into a clarithromycin-susceptible strain, *H. pylori* UA802, by natural transformation followed by characterization of their effects on MLS resistance in an isogenic background. Strains with A-to-G and A-to-C mutations at the same position within the 23S rRNA gene had similar levels of clarithromycin resistance, and this level of resistance was higher than that for strains with the A-to-T mutation. Mutations at position 2142 conferred a higher level of clarithromycin resistance than mutations at position 2143. All mutations at position 2142 conferred cross-resistance to all MLS antibiotics, which corresponds to the type I MLS phenotype, whereas mutations at position 2143 were associated with a type II MLS phenotype with no resistance to streptogramin B. To explain that A-to-G transitions were predominantly observed in clarithromycin-resistant clinical isolates, we propose a possible mechanism by which A-to-G mutations are preferentially produced in *H. pylori*.

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Helicobacter pylori is a microaerophilic, gram-negative bacterium that colonizes the human gastric mucosa and that causes gastritis and peptic ulceration (8). It is also associated with the development of gastric cancer (22). Clarithromycin is a potent macrolide that has frequently been used in combination with other antimicrobial agents for the treatment of *H. pylori* infections (23, 32). However, the development of clarithromycin resistance among *H. pylori* strains has become a predominant cause of the failure of therapy incorporating clarithromycin (3, 15). Previous studies have examined clarithromycin-resistant *H. pylori* isolates from various geographic locations and have revealed that mutations responsible for alterations in the 23S rRNA gene are the mechanism of clarithromycin resistance (7, 21, 28, 29, 34, 35). Specifically, adenine-to-guanine transitions at either position 2058 or position 2059 (*Escherichia coli* coordinates) in the peptidyltransferase region of the 23S rRNA were in most cases associated with clarithromycin resistance. Recently, two identical copies of the 23S rRNA have been sequenced and the transcription start site of the gene from a clarithromycin-susceptible strain, strain UA802, was determined (30). According to the new numbering scheme for *H. pylori* 23S rRNA, *E. coli* bases 2058 and 2059 correspond to *H. pylori* positions 2142 and 2143, respectively (see Fig. 3).

In *E. coli*, as well as in some other bacteria, it is well known that the base equivalent to base A2058 in the 23S rRNA of *E. coli* is the target of ribosomal methyltransferase (products of *erm* genes which are frequently plasmid encoded) and the binding site for macrolide antibiotics (5, 39). Methylation or mutation at this position confers complete cross-resistance to the macrolide, lincosamide, and type B streptogramin (MLS) antibiotics (MLS resistance), suggesting that these structurally distinct antibiotics have similar effects in inhibiting ribosomal function. Mutations within the vicinity, at position 2059 or 2057, have also been associated with resistance to the macrolide group of antibiotics (20, 24, 33). To date, the MLS resistance phenotypes associated with mutations in the peptidyltransferase region of the 23S rRNA have not yet been investigated in *H. pylori*.

This study was initiated to characterize the MLS phenotypes and the associated genotypes of several clinical isolates of *H. pylori*. Furthermore, to demonstrate the cause-effect relationship between particular types of mutations and MLS resistance phenotypes, we performed in vitro site-directed mutagenesis. The site-directed point mutations in the 23S rRNA gene were introduced into an MLS-susceptible strain of *H. pylori* by natural transformation, followed by characterization of their effects on MLS resistance in an isogenic background.

MATERIALS AND METHODS

H. pylori strains, growth medium, and antibiotics. *H. pylori* A, B, D, E, and M2 are clarithromycin-resistant clinical isolates which originated in Europe (30). Clarithromycin-susceptible strain UA802 was an isolate from the University of Alberta Hospital and has been used extensively in this laboratory (16). *H. pylori*

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MLS RESISTANCE MUTATIONS IN *H. PYLORI* 1953

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TABLE 1. Oligonucleotides used in this study

Designation	Sequence (5' to 3') ^a	Position ^b	Strand ^c	Purpose ^d	Reference or source
DP1	ACGGCGGCCGTAACTATA	1985-2002	+	Commonly used for SDM, sequencing	30
ZGE23	ACAGGGCACTTAGCTA	2292-2277	-	Commonly used for SDM, sequencing	30
GW1	GACGGGAAGACCCCGTGG	2137-2155	+	SDM (A2142G)	This work
GW11	GGTCCTTCCGCTTGC	2148-2130	-	SDM (A2142G)	This work
GW2	GACGGCAAGACCCCGTGG	2137-2155	+	SDM (A2142C)	This work
GW12	GGTCCTTCCGCTTGC	2148-2130	-	SDM (A2142C)	This work
GW3	GACGGTAAGACCCCGTGG	2137-2155	+	SDM (A2142T)	This work
GW13	GGTCCTTACCGCTTCCG	2148-2130	-	SDM (A2142T)	This work
GW4	CACGGAGAGACCCCGTGG	2137-2155	+	SDM (A2143G)	This work
GW14	GGTCCTTCCGCTTGC	2148-2130	-	SDM (A2143G)	This work
GW5	GACGGACAGACCCCGTGG	2137-2155	+	SDM (A2143C)	This work
GW15	GGTCCTGTCGGCTTGC	2148-2130	-	SDM (A2143C)	This work
GW6	GACGGATAGACCCCGTGG	2137-2155	+	SDM (A2143T)	This work
GW16	GGTCATCCGCTTGC	2148-2130	-	SDM (A2143T)	This work
GW7	AGACCAAAAGACCCCGTGG	2136-2154	+	SDM (G2141A)	This work
GW17	GTCCTTTCGCTTGC	2147-2129	-	SDM (G2141A)	This work

^aThe bases different from those in the wild-type 23S rRNA gene sequence are underlined.^bPositions refer to the new numbering scheme for the *H. pylori* 23S rRNA (30).^cThe plus (+) strand is equivalent to the rRNA sequence, and the minus (-) strand is the complementary one.^dSDM, site-directed mutagenesis.

h. pylori strains were grown on BH-YE agar (3.7% brain heart infusion agar base with 0.3% yeast extract and 5% animal serum) at 37°C under microaerobic conditions (5% CO₂, 5% H₂, and 90% N₂). The antibiotics used in this study were obtained as follows: Clarithromycin was from Bayer, Leverkusen, Germany; clindamycin was from the Upjohn Company of Canada, Don Mills, Ontario, Canada; and quinupristin (streptogramin B) and dalfopristin (streptogramin A) were provided by both Sylvia Pong-Porter (Department of Microbiology, Mount Sinai Hospital, Toronto, Ontario, Canada) and Rhone-Poulenc Rorer, Collegeville, Pa.

MIC test. *H. pylori* cells were grown for 2 days and were suspended in sterile BH-YE liquid medium, and the turbidity of the suspension was adjusted to that of a 2.0 McFarland standard. The suspended cells were inoculated (8 µl/spot) of a 2.0 McFarland standard. The suspended cells were inoculated (8 µl/spot) onto BH-YE agar plates containing different concentrations of antibiotics obtained by serial twofold dilution. The plates were incubated as described above, and the growth was examined after 3 days.

DNA manipulation. Chromosomal DNAs from the *H. pylori* strains were isolated by a previously described method (13). DNA sequencing was carried out with the thermocycling sequencing system with Thermo-Sequenase purchased from Amersham Life Sciences, Cleveland, Ohio. Other DNA manipulations including PCR and gel electrophoresis were performed by standard methods (25).

Site-directed mutagenesis. A series of point mutations at position 2142, 2143, or 2141 of the *H. pylori* 23S rRNA gene were generated by a sequential PCR method (1). Table 1 lists all of the primers that were used. To create a particular point mutation, two common primers, primers DP1 and ZGE23, and a pair of primers containing site-specific mutation were used (Fig. 1). For example, to make an A2142G mutation, two fragments were amplified by PCR with primer pairs DP1-GW11 and ZGE23-GW1 in the first step. In the second PCR step, these two fragments encompassing the mutation were annealed with each other and were extended by mutually primed synthesis. The final products were 307-bp PCR fragments containing a point mutation in the center. The PCR products were gel purified with Spin-X (purchased from Corning Costar Corporation, Cambridge, Mass.), and the site-directed mutations in the PCR fragments were verified by DNA sequencing.

Natural transformation. The PCR fragments containing a site-specific mutation were introduced into clarithromycin-susceptible strain *H. pylori* UA802 by natural transformation as described previously (15), and the transformants were selected for clarithromycin resistance. Briefly, recipient cells were heavily inoculated on cold BH-YE agar plates and were grown for 5 h, followed by the addition of 0.2 to 0.5 µg of DNA (307-bp PCR fragment) onto the bacterial lawn. After incubation for 16 to 24 h under microaerobic conditions the transformed cells were streaked onto BH-YE agar plates containing the selective antibiotic (2, 0.5, 0.1, or 0.02 µg of clarithromycin per ml), and the transformants (single colonies) were obtained after incubation for 3 to 4 days.

RESULTS

Characterization of MLS phenotypes of clinical *H. pylori* isolates and association with specific mutations in the 23S rRNA gene. To test the MLS phenotypes of several clarithromycin-resistant *H. pylori* strains, clarithromycin, clindamycin,

and quinupristin were used as the representative antibiotics for macrolide, lincosamide, and type B streptogramin, respectively. On the basis of the MICs of the three antibiotics presented in Table 2, two phenotypes of MLS resistance were identified. Strains that are highly resistant to clarithromycin (MICs, ≥ 8 µg/ml) exhibit high-level resistance to clindamycin (MICs, > 512 µg/ml) and quinupristin (MIC, 128 µg/ml) (type I). Other strains, strains A and B, for which the MIC of clarithromycin ranged from 1 to 4 µg/ml (intermediate level of resistance) have intermediate-level resistance to clindamycin (MIC, 256 µg/ml) but minimal resistance to quinupristin (MIC, 4 µg/ml), which is identical to that for susceptible strain UA802 (type II).

To examine the genetic basis of these MLS phenotypes, we determined the nucleotide sequence of the 23S rRNA gene coding for the peptidyltransferase region of the 23S rRNA from all of these strains. Briefly, a PCR fragment was amplified from the chromosomal DNA with primers DP1 and ZGE23 (Table 1), followed by sequencing of the fragment with primer DP1. By comparing the DNA sequences from different strains, specific mutations at position 2142 or 2143 were associated with the two MLS phenotypes: A2142G for type I MLS resistance and A2143G for type II MLS resistance (Table 2).

In addition to the mutation at position 2142 or 2143, other mutations were observed in certain strains (in *H. pylori* coordinates, A2085G in strain A and A2223G in strain E). Notably, although strains A and B have the same phenotypic resistance to clindamycin and quinupristin, there are minor but significant differences in the MICs of clarithromycin for the two strains (4 versus 1 µg/ml; Table 2). To find out whether the additional mutation accounts for the observed difference in the MICs of clarithromycin for strains A and B, we introduced the PCR fragments containing the relevant mutations amplified with primer pair DP1-ZGE23 from the chromosomal DNA of strain A or B into *H. pylori* UA802. The relevant mutations in both transformants were confirmed by DNA sequencing to be the same as those in the donor strain. By examining their effects in an isogenic background, it was found that the MIC of clarithromycin was the same for both transformants (4 µg/ml). Therefore, it is likely that the observed difference in the clarithromycin MICs for strains A and B is not due to the additional A2085G mutation but, rather, reflects other host effects.

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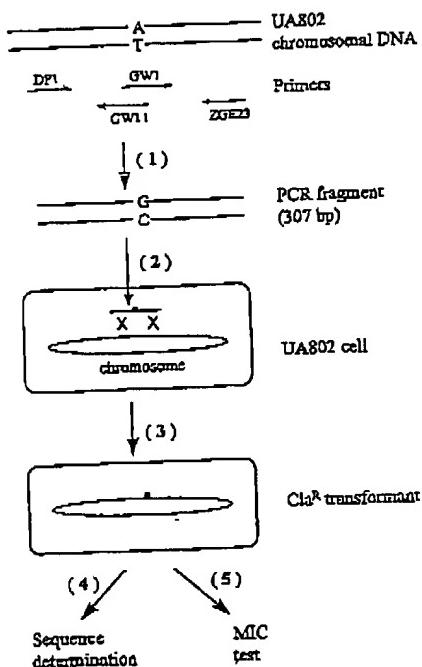


FIG. 1. Outline of procedures for construction and characterization of site-directed mutations. The steps are numbered in series. (Step 1) Site-directed mutagenesis. Wild-type *H. pylori* UA802 chromosomal DNA was used as the template for sequential PCR. The figure shows an example for constructing an A2142G mutation, and the primers used (Table 1) are indicated. (Step 2) Natural A2142G mutation, and the primers used (Table 1) are indicated. (Step 3) Selection for clarithromycin resistance. This step includes 3 to 4 days of incubation for the formation of single colonies and subsequent subculturing to obtain Clr^R genetically stable Clr^R transformants. (Step 4) Genotypic identification of Clr^R mutants by DNA sequencing. (Step 5) Characterization of MLS phenotypes of Clr^R mutants by the MIC test.

The additional mutations observed in the present study seem to be unrelated to clarithromycin resistance but, rather, represent microdiversity in the sequences of 23S rRNA genes from different *H. pylori* strains.

Synergistic effect of type A and B streptogramins on *H. pylori*. It has been shown in *E. coli* and some other bacteria that type A and B streptogramins can block the peptidyltransferase activity of the 50S ribosomal subunit and can have synergistic effects resulting from conformational changes imposed upon the peptidyltransferase center by streptogramin A and by inhibition of both early and late stages of protein synthesis (4). For the *H. pylori* strains mentioned above, we also characterized the MICs of streptogramin A (dalofopristin) and a mixture of streptogramin B and A (quinupristin-dalopristin in a 30:70 ratio; RP59500) (2). All the strains tested in this study were inhibited by 8 µg of dalopristin per ml (Table 2), regardless of their susceptibility or resistance to MLS antibiotics. By using RP59500, the MICs for all the strains further decreased to 0.5 to 2 µg/ml (Table 2), demonstrating a synergistic effect of streptogramins A and B on *H. pylori*. These effects are similar to those previously observed for both *Staphylococcus aureus* (12) and *Enterococcus faecium* (11).

In vitro site-directed mutagenesis in the 23S rRNA gene. Of all the clarithromycin-resistant clinical isolates of *H. pylori* re-

ported so far, the resistance in most strains is associated with A-to-G transition mutations (at position 2142 or 2143, according to the revised numbering system; see Fig. 3) in the 23S rRNA gene (7, 21, 28, 29, 34, 35). To find out if other types of mutations (A to C or A to T) also confer clarithromycin resistance, we performed *in vitro* site-directed mutagenesis. Since a suitable replicative or integrative vector is generally unavailable for *H. pylori*, we used the natural transformation process to introduce site-directed mutations into the chromosome. This corresponds to the situation of clarithromycin resistance in clinical isolates in which mutations are chromosomal rather than plasmid borne. The natural transformation process includes DNA uptake and homologous DNA recombination (Fig. 1). After clarithromycin selection, only those mutations that confer clarithromycin resistance and that are incorporated into the chromosome can give rise to transformants. In addition, we took advantage of the low fidelity of *Taq* DNA polymerase for use in sequential PCR to construct mutants with site-directed mutations. Using a strategy similar to that described by Kok et al. (17), we sought to obtain other types of mutations (random mutations) that confer Clr^R and that may be screened out by natural transformation. Using the primers listed in Table 1 and the method described in Materials and Methods, we obtained the PCR fragments containing the following specific point mutations: A2142G, A2142C, A2142T, A2143G, A2143C, A2143T, and G2141A. These were verified by DNA sequencing. Note that at this stage a very minor fraction of the PCR products may possibly contain certain random mutations, produced by *Taq* DNA polymerase errors, that could not be detected by DNA sequencing. These site-specific mutations were introduced into UA802 by natural transformation so that we could characterize their effects on MLS resistance in an isogenic background.

First, different concentrations of clarithromycin (2, 0.5, 0.1, and 0.02 µg/ml in agar medium) were tested for transformant selection. We found that 0.1 µg/ml is the lowest concentration that can be used to give unambiguous transformation results. With this concentration of antibiotic, no transformants were obtained for the control (wild-type) DNA or for DNA with the A2143T or G2141A mutation (Table 3), suggesting that these mutations do not confer any resistance to up to 0.1 µg of clarithromycin per ml. In contrast, more than 1,000 transfor-

TABLE 2. Mutations in the 23S rRNA gene and associated MLS phenotypes of clinical *H. pylori* isolates

<i>H. pylori</i> strain ^a	23S rRNA gene mutation	MIC (µg/ml) ^b					MLS pheno-type ^c
		Cla	Cln	Qnp	Dip	RP59500	
UA802	Wild type	0.004	32	4	8	2	S
A	A2143G	4	256	4	8	2	R-II
B	A2143G	1	256	4	8	0.5	R-II
D	A2142G	8	>512	64	8	1	R-I
MQ	A2142G	16	>512	128	8	1	R-I
E	A2142G	16	>512	128	8	2	R-I
Ery-1	A2142G	16	>512	128	8	2	R-I
Clr ^R -1	A2142G	16	>512	128	8	2	R-I

^a Ery-1 and Clr^R-1 are erythromycin- and clarithromycin-resistant strains of UA802, respectively, obtained by transformation of the erythromycin or clarithromycin resistance determinant from strain E (30).

^b Abbreviations for antibiotics: Cla, clarithromycin; Cln, clindamycin; Qnp, quinupristin (streptogramin B); Dip, dalopristin (streptogramin A); RP59500, mixture of quinupristin and dalopristin.

^c S, susceptible; R-II, type II resistance; R-I, type I resistance.

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TABLE 3. In vitro site-directed mutagenesis of *H. pylori* 23S rRNA gene and MLS phenotypes of the mutants obtained

Mutation in PCR fragment	No. of transformants obtained	No. of transformants sequenced	Mutation in 23S rRNA gene	MIC ($\mu\text{g/ml}$) ^a		
				Cla	Cln	Qap
Wild type	0		Wild type	0.004	32	4
G2141A	0			NK	NK	NK
A2142G	>1,000	8	A2142G	16	>512	128
A2142C	>1,000	3	A2142C	16	>512	128
A2142C ^b		1	A2142C/T	8	>512	128
A2142T	>1,000	4	A2142T	4	512	64
A2143G	>1,000	4	A2143G	4	256	4
A2143C	>1,000	3	A2143C	4	256	4
A2143C ^c		1	A2143C + A2142G	16	>512	128
A2143T	0			NK	NK	NK

^a Abbreviations: Cla, clarithromycin; Cln, clindamycin; Qap, quinupristin; NK, not known.^b Of the four transformants picked up for sequencing, one contained an A2142C/T mutation (both C and T bands of equal intensities were revealed on the sequencing gel [see Fig. 2]).^c Of the four transformants picked up for sequencing, one contained a double mutation (A2143C and A2142G).

ments with each of the A2142G, A2142C, A2142T, A2143G, and A2143C mutations were obtained. This corresponds to a transformation frequency of 10^{-5} per viable cell. Subsequently, some of the transformants were colony purified. Upon subculturing of the colonies, the Cla^r phenotypes of these clones were shown to be stable, suggesting that the corresponding mutations were incorporated into the chromosome.

Next, the chromosomal DNA sequences of the region of interest were examined for some of these Cla^r clones (Fig. 2; Table 3). In the majority of the clones examined, targeted mutations were detected, i.e., substitution of G, C, or T for A2142 and substitution of G or C for A2143. Two untargeted mutations were observed. One was a double mutation at A2143C and A2142G which was obtained from transformation of the A2143C mutation. Another clone obtained from the transformation of A2142C was shown to carry an A2142C/T mutation because both C and T bands at position 2142 were revealed on the sequencing gel at approximately the same intensity (Fig. 2). Since there are two copies of the 23S rRNA gene in *H. pylori* (16, 30, 31), this mutant may represent a heterozygote with C in one copy of the gene and T in the other copy of the gene. The occurrence of the mutants with untargeted mutations may be due to (i) a mutation randomly introduced in the PCR fragment or (ii) a spontaneous or drug-induced mutation which occurred in recipient cells.

Characterization of MLS phenotypes of the site-directed mutations. The MICs of clarithromycin, clindamycin, and quinupristin for all the constructed mutants with site-directed mutations are listed in Table 3, from which the following points can be made. (i) Mutations at position 2142 always confer a higher level of clarithromycin resistance than mutations at position 2143. At the same position, an A-to-G or an A-to-C mutation gives rise to similar clarithromycin MICs which are higher than that conferred by the A-to-T mutation. (ii) There is no further increase in the MIC of clarithromycin for the mutant with the double mutation (A2142G plus A2143C) compared to that for the mutant with the single mutation (A2142G) (MIC, 16 $\mu\text{g/ml}$). The clarithromycin MIC for heterozygous mutant A2142C/T is 8 $\mu\text{g/ml}$, which is intermediate between those for both homozygous mutants, A2142C (MIC, 16 $\mu\text{g/ml}$) and A2142T (MIC, 4 $\mu\text{g/ml}$). (iii) Two kinds of MLS phenotypes were observed for these mutants with site-directed mutations (summarized in Fig. 3). Any mutation (A to G, C, or T) which occurred at position 2142 conferred cross-resistance to all three kinds of antibiotics tested (type I MLS resistance). Substitution of A at position 2143 with G or C gave rise to

intermediate levels of resistance to clarithromycin and clindamycin but no resistance to quinupristin (type II MLS resistance). These two phenotypes of MLS resistance are similar to those observed for clinical isolates (Table 2), but distribution of the 23S rRNA genotypes associated with these phenotypes in the constructed mutants are more extensive than those found in clinical isolates, in which A-to-G mutations have predominantly been observed, A-to-C mutations have occasionally been observed, and A-to-T mutations have never been observed.

DISCUSSION

In the first part of this study, we observed the following two major phenotypes of MLS resistance for *H. pylori*: type I, high-level cross-resistance to all MLS antibiotics, and type II, intermediate-level resistance to clarithromycin and clindamycin but no resistance to streptogramin B. By examining the DNA sequences of these strains, specific mutations in the 23S rRNA gene, A2142G and A2143G, were associated with these two MLS phenotypes, respectively. The observation that the A2142G mutation is associated with cross-resistance to all MLS antibiotics is in agreement with the finding for *E. coli* (equivalent to the A2058G mutation) and other organisms (27,

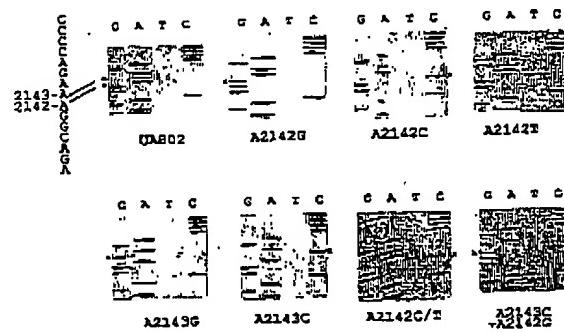


FIG. 2. Nucleotide sequences of the short region in the 23S rRNA gene from Cla^r *H. pylori* UAB02 (wild type) and the constructed Cla^r mutants showing their relevant genotypes. The corresponding sequence for the wild-type strain (see Fig. 3) is indicated on the left, with both adenines at positions 2142 and 2143 highlighted with black dots. The position of a specific base substitution(s) in each particular mutant is marked with an asterisk.

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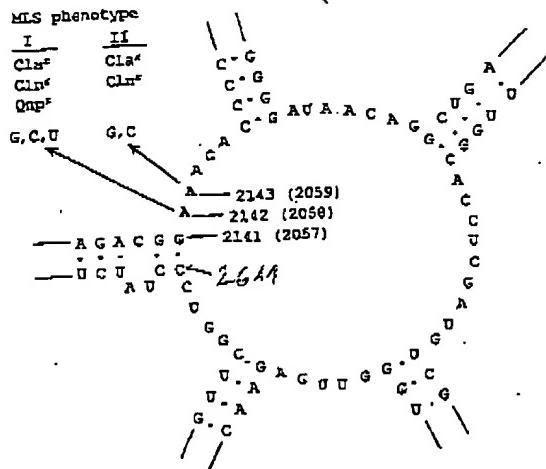


FIG. 3. Secondary structure of the central part of domain V (peptidyltransferase loop) of the *H. pylori* 23S rRNA gene based on the model of Egebjerg et al. (9), with indication of the mutations that confer MLS resistance. The mutation sites are numbered according to the newly proposed numbering system (30), and the equivalent positions in *E. coli* are indicated in parentheses. The base substitutions made by site-directed mutagenesis in this work are indicated by arrows, and the associated MLS phenotypes are indicated. Abbreviations for antibiotics: Cla, clarithromycin; Cln, clindamycin; Qnp, quinupristin.

36, 39). The A-to-G mutation at the base equivalent to base 2059 in *E. coli* has been shown to be associated with resistance to macrolides in many organisms including mycoplasmas, mycobacteria (18, 19, 37), and *H. pylori*, but its association with cross-resistance to all MLS antibiotics was not reported. A recent study with propionibacteria demonstrated that the A2059G mutation gives rise to a high level of resistance to macrolides, a moderate level of resistance to lincosamides, and no resistance to type B streptogramins (24). Similar to the findings in that study, we observed that the A2143G mutation in the *H. pylori* 23S rRNA gene is linked to an intermediate level of resistance to clarithromycin and clindamycin and no resistance to streptogramin B.

In addition, we also determined the MICs of dalfopristin and a combination of quinupristin and dalfopristin (RP59500) and demonstrated a synergistic effect of type A and B streptogramins on *H. pylori*. Both MLS-sensitive and MLS-resistant *H. pylori* strains were found to be moderately susceptible to dalfopristin and susceptible to RP59500. RP59500 is a new semisynthetic injectable streptogramin which has been shown to have excellent activity against most gram-positive bacteria including staphylococci, *E. faecium*, and pneumococci (2). It offers some advantages over the commercially available antimicrobial agents against drug-resistant gram-positive bacteria. In vitro studies have shown that it also has good activity (MIC, <2 µg/ml) against some selected gram-negative pathogens such as *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, and *Neisseria gonorrhoeae* and a moderate level of activity against *Haemophilus influenzae* (MICs, 2 to 8 µg/ml) (for a review, see reference 2). Our results showed that RP59500 has good activity (MICs, 0.5 to 2 µg/ml) against *H. pylori* and even against MLS-resistant strains. Thus, our data may prompt consideration of the use of quinupristin-dalfopristin as a possible alternative antibiotic in the case of failure of therapy with a clarithromycin-based treatment regimen.

The major goal of this work was the construction of a series

of site-directed mutations in the *H. pylori* 23S rRNA gene that are associated with clarithromycin resistance and the characterization of their effects on MLS resistance. At first, by introducing a 307-bp PCR fragment containing a specific point mutation into UA802 by natural transformation, a reasonably high efficiency of transformation was obtained, suggesting that a DNA fragment as small as 300 bp is sufficient for the occurrence of a double crossover in homologous recombination in *H. pylori* (Fig. 1). Using this method, we have constructed the expected mutants except mutant A2143T with base substitutions at position 2142 or 2143. In addition, a double point mutation and a heterozygous mutant (A2142C/T) were obtained. By examining the MLS phenotypes of these constructed mutants, two types of MLS resistance similar to those seen for the clinical isolates were observed. Type I is associated with mutations at position 2142 and type II is associated with mutations at position 2143 (Fig. 3). These data imply that position 2142 is the binding site for all MLS antibiotics and that position 2143 has a binding affinity for macrolides and lincosamides but not for streptogramin B. According to these results, we can consider the different MLS phenotypes as a signature for the specific type of mutation in the 23S rRNA gene in identifying clarithromycin-resistant *H. pylori* isolates.

G2057A in *E. coli* conferred low-level resistance to erythromycin (10). A similar result was found recently with propionibacteria (24). It was hypothesized that disruption of the G2057-C2611 base pairing leads to a weaker rearrangement and affects the binding site of macrolide antibiotics (33). To investigate whether there is a similar effect in *H. pylori*, we also created an equivalent mutation, G2141A. However, after selection with 0.1 µg of clarithromycin per ml, no transformant was obtained. Under identical transformation conditions, the other mutations gave rise to more than 1,000 transformants. The unavailability of G2141A and A2143T mutants gave us at least indirect evidence that such mutations do not confer a significant level of clarithromycin resistance. We also attempted to use even lower concentrations of clarithromycin (0.02 µg/ml) for the selection of resistance with the goal of obtaining these two mutants, but they were not forthcoming.

Another interesting feature that we noted is that most mutants constructed appear to carry a homozygous mutation in both copies of the 23S rRNA gene, because only a single band representing the mutated base was revealed at the relevant position on a sequencing gel (Fig. 2). Certainly, the evidence from sequencing alone is not convincing for the resolution of heterozygosity. Recently, Sander et al. (26) demonstrated that clarithromycin resistance is dominant over sensitivity in *Mycobacterium smegmatis*, another eubacterium carrying two rRNA operons. Evidence of heterozygosity has also been reported for a few clinical isolates of *H. pylori* (28, 34). However, most of the Cla^r *H. pylori* isolates so far reported are homozygous mutants. Thus, it is possible that the minor fraction of heterozygous mutants escaped our examination since only small numbers of transformants (four clones for each type of mutation) were sequenced. The prevalence of homozygosity over heterozygosity in *H. pylori* may reflect a high efficiency of DNA recombination in this organism. The mutation in one copy of the 23S rRNA gene may be easily copied to the other 23S rRNA gene by efficient homologous DNA recombination under the selection pressure to produce a diploid mutation that may confer a higher level of resistance.

To date, A-to-G mutations have been predominantly associated with clarithromycin resistance in clinical *H. pylori* isolates; few mutations from A to C and no mutation from A to T in the 23S rRNA gene were identified (7, 21, 28, 29, 30, 34, 35). Concerning the possible mechanism for this phenomenon

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in *H. pylori*, Debets-Ossenkopp et al. (6) proposed that it is due to the relatively higher growth rates and the MIC for the strains with A-to-G mutations. Indeed, our preliminary data suggest that the growth of the A-to-C or A-to-T mutants is significantly slower (a lag of about 1 day) than that of the wild type or the A-to-G mutants (38). The differences in the MIC of clarithromycin that we observed for these mutants ($G = C > T >> A$) are essentially in agreement with their data (6). However, we found that the A-to-G and the A-to-C mutations at the same position mediate identical MICs; and particularly, the MIC for the strains with the A2142C mutation is higher than that for the strains with the A2143G mutation. We observed that two additional mutations in the 23S rRNA gene from Claf^r clinical isolates are also A-to-G transitions but are unrelated to clarithromycin resistance. By inference from other gene sequences in *H. pylori*, such as those for α 1,3-fucosyltransferase genes (14, 31), we also found that transitions (from the A/T base pair to the G/C base pair or vice versa) account for the majority of intraspecies microdiversity. In general, a particular mutation occurs in two steps: mutation formation and selective accumulation. As mentioned above (6), the relatively higher growth rate and MIC could offer the A-to-G mutation an advantage over other types of mutations in selective accumulation (step 2). As an additional possible mechanism, we propose that the A-to-G transitions may be preferentially formed or produced (in step 1) in *H. pylori*.

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Macrolide Resistance in *Helicobacter pylori*: Rapid Detection of Point Mutations and Assays of Macrolide Binding to Ribosomes

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Resistance of *Helicobacter pylori* to macrolides is a major cause of failure of eradication therapies. Single base substitutions in the *H. pylori* 23S rRNA genes have been associated with macrolide resistance in the United States. Our goal was to extend this work to European strains, to determine the consequence of this mutation on erythromycin binding to *H. pylori* ribosomes, and to find a quick method to detect the mutation. Seven pairs of *H. pylori* strains were used, the parent strain being naturally susceptible to macrolides and the second strain having acquired an in vivo resistance during a treatment regimen that included clarithromycin. The identity of the strains was confirmed by random amplified polymorphic DNA testing with two different primers, indicating that resistance was the result of the selection of variants of the infecting strain. All resistant strains were found to have point mutations at position 2143 (three cases) or 2144 (four cases) but never on the opposite DNA fragment of domain V of the 23S rRNA gene. The mutation was A → G in all cases except one (A → C) at position 2143. Using *Bsa*I and *Bbs*I restriction enzymes on the amplified products, we confirmed the mutations of A → G at positions 2144 and 2143, respectively. Macrolide binding was tested on purified ribosomes isolated from four pairs of strains with [¹⁴C]erythromycin. Erythromycin binding increased in a dose-dependent manner for the susceptible strain but not for the resistant one. In conclusion we suggest that the limited disruption of the peptidyltransferase loop conformation, caused by a point mutation, reduces drug binding and consequently confers resistance to macrolides. Finally, the macrolide resistance could be detected without sequencing by performing restriction fragment length polymorphism with appropriate restriction enzymes.

Helicobacter pylori, a gram-negative bacterium which colonizes the human stomach, has been the focus of important research in recent years. *H. pylori* infection induces chronic gastritis (16) which can lead to duodenal and gastric ulcers (19) as well as to malignancies such as gastric carcinoma and lymphoma (9). The importance of eradication of the bacteria and the resulting cure of peptic ulcer disease was confirmed at a Consensus Conference organized by the National Institutes of Health in 1994 (23). More recently a European Consensus report recommended a triple therapy, associating a potent anti-secretory drug with two antibiotics, as the first-choice treatment (8).

Clarithromycin is a component of the most widely used regimens. However, macrolide resistance in *H. pylori* has been shown to occur at different rates (1 to 10%) in different countries (17) and is an important cause of the failure of these regimens. Moreover, *H. pylori* mutants resistant to macrolides are easily obtained by *in vitro* selection (12). Macrolides inhibit protein synthesis, stimulating dissociation of peptidyl-tRNA from the ribosome during the elongation reaction (21).

Macrolide resistance is due to several mechanisms including lack of macrolide binding to the ribosome target and, to a lesser extent, macrolide inactivation by enzymes, impermeability of the bacterial membrane, and active drug efflux (35). This last mechanism is apparently of importance in *Streptococcus pyogenes* and *Streptococcus pneumoniae* (4, 26, 30). The target modification usually involves a posttranscriptional modifica-

tion or a mutation situated in the peptidyltransferase domain (domain V) of the 23S rRNA (14), while mutations of the ribosomal proteins have also been described (35). More recently, macrolide resistance in *Escherichia coli* (27, 34), *Mycoplasma pneumoniae* (15), *Mycobacterium intracellulare* (20), and *Mycobacterium avium* (22) has also been linked to a point mutation in the 23S rRNA. This mutation (A → G at positions 2143 or 2144, formerly labelled 2058 and 2059, respectively) was found for the first time by Versalovic et al. in *H. pylori* strains isolated in the United States (33).

The aim of this study was to test the consequences of these mutations on macrolide binding to *H. pylori* ribosomes and to develop a method which would be easier than sequencing to detect these mutations.

MATERIALS AND METHODS

Bacterial strains. The strains were isolated from patients consulting at St. André Hospital, Bordeaux, France, who suffered from duodenal ulcer disease or nonulcer dyspepsia. Gastric biopsies were grown and then cultured on Wilkins Chalgren agar (Oxoid, Basingstoke, United Kingdom) enriched with 10% human blood and containing vancomycin (10 mg/liter), cefotaxime (2 mg/liter), trimethoprim (5 mg/liter), and cycloheximide (Act-Dione; 100 mg/liter) and on pylon agar (bioMérieux, Marcy-l'Etoile, France). Plates were incubated for 3 to 7 days in microaerobic atmosphere at 37°C. Identification at the species level was based on morphology and the presence of oxidase, catalase, and urease. Seven pairs of *H. pylori* strains, in addition to reference strain CIP 101260 from the Collection de l'Institut Pasteur, were included in this study. For each pair of strains, the second strain was isolated at a follow-up visit after an unsuccessful treatment that included clarithromycin.

Determination of MICs. MICs of clarithromycin were determined by the standard agar dilution technique. Briefly, a suspension of each strain (approximately 10⁶ CFU/ml) was inoculated, with a Steers apparatus (28), onto plates containing Wilkins Chalgren agar enriched with 10% sheep blood and Polyvinyl, as well as clarithromycin at concentrations ranging from 0.0035 to 128 µg/liter. Incubation was performed in a microaerobic atmosphere for 48 h at 37°C.

The strains were also screened for resistance to other macrolides including

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TABLE 1. Agar dilution MICs of clarithromycin and mutations of *H. pylori* isolates investigated

Patient or strain	Date of specimen collection (day/mo/yr)	Isolate	Clarithromycin MIC (μ g/ml)	23S rRNA domain V
Ia	02/07/92	594S	0.06	Wild type
Ib	23/10/92	677R	32	2144A→G*
IIa	07/09/92	638S	0.06	Wild type
IIb	19/10/92	675R	32	2144A→G
IIIa	14/09/92	646S	0.06	Wild type
IIIb	04/11/92	683R	16	2143A→G
IVa	30/04/93	764S	0.06	Wild type
IVb	11/06/93	782R	64	2144A→G
Va	02/08/93	803S	0.06	Wild type
Vb	05/10/95	825R	>128	2143A→C
VIIa	22/11/93	848S	0.03	Wild type
VIIb	02/01/95	1060R	>128	2143A→G
VIIa	12/12/94	1054S	0.125	Wild type
VIIb	16/01/95	1069R	16	2144A→G
CIP 101260				Wild type

* 2144A→G, A→G mutation at position 2144.

14-carbon ring (erythromycin, roxithromycin), 15-carbon ring (aztreomycin), 16-carbon ring (spiramycin) compounds and related compounds; lincosamides (clavamycin, clindamycin); and streptogramins (pristinamycin), as well as tetracycline and chloramphenicol. The two components of streptogramins were tested together. Susceptibility testing was performed by the E test (AB test) (AB Biostick, Solna, Sweden) except for testing of spiramycin, lincomycin, and pristinamycin for which only disks (Sanofi Diagnostics Pasteur, Marne-la-Coquette, France) were used.

Random amplified polymorphic DNA (RAPD) testing. Genomic DNA was isolated from lyzed *H. pylori* cells following phenol-chloroform extraction and ethanol precipitation as described previously (13). Amplification was carried out in a 25- μ l volume containing 1 μ g of *H. pylori* genomic DNA, 67 mM Tris-HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01% Tween 20, 1.5 mM MgCl₂, a 0.4 mM concentration of decoxynucleoside triphosphate mixture, a 5 μ M concentration of primer, 1 U of Taq DNA polymerase (Eurobio, Les Ulis, France), and sterilized water. Each reaction mixture was overlaid with 50 μ l of mineral oil. Two primers were used: 3881 (5'-AAC GCG CAA C-3') (1) and OPB8 (5'-GAA ACA CCC C-3') (Bioprobe Systems, Montrouge, France). A Perkin-Elmer (Foster City, Calif.) 480 thermal cycler was used for amplification. The cycling program was composed of 1 cycle at 94°C for 10 min; 40 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 1 min; and a final incubation at 72°C for 10 min. Then, 20- μ l aliquots of PCR products were separated by electrophoresis in 1.5% agarose gels containing 1 μ g of ethidium bromide per ml, and the gel was photographed under UV light. The DNA of *Xba*II was used as a size marker in all gels under UV light. The DNA of *Xba*II was used as a size marker in all gels under UV light. The DNA of *Xba*II was used as a size marker in all gels under UV light.

Detection of mutations. (i) PCR amplification. Two pairs of primers were used to amplify two fragments of the peptidyltransferase region of the 23S rRNA. The sequences of the primers were based on the published sequence of the 23S ribosomal DNA gene of *H. pylori* (GenBank accession no., U27270). A primer extending from position 1820 to 1839 (5'-CCA CAG CGA TGT GGT CTC AG-3') and a reverse primer from position 2244 to 2275 (5'-CTC CAT AAG AGC CAA ACC CC-3') were used to amplify a fragment (A) of 425 bp. A primer from position 2415 to 2433 (5'-GCA CAA GCC AGC CTG ACT G-3') and a reverse primer from position 2828 to 2810 (5'-AGC AGT TAT CAC ATC CCT G-3') were chosen to amplify fragment (B) of 414 bp. PCR amplification of DNA was performed in a final volume of 100 μ l containing 1 μ g of *H. pylori* genomic DNA, 67 mM Tris-HCl (pH 8.8), 16 mM (NH₄)₂SO₄, 0.01% Tween 20, 1.5 mM MgCl₂, a 0.2 nM concentration of decoxynucleoside triphosphate mixture, 1 μ M concentrations of primers and 2 U of Taq DNA polymerase. Each reaction mixture was overlaid with 50 μ l of mineral oil. The cycling program was 1 cycle at 94°C for 10 min; 30 cycles of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The two reactions resulted in a single fragment of the expected size.

(ii) Sequencing of domain V of the 23S rRNA gene. Before sequencing, PCR products (200 to 300 μ l from each strain) were prepared by purification and concentration with Wizard PCR Prep columns (Promega, Madison, Wis.) and were resuspended in 50 μ l of H₂O. The same primers used for PCR amplification of fragments A and B were used for sequencing. Sequencing was performed on the two strands of each amplicon with an automated DNA sequencer and the *Taq* DyeDeoxy terminator cycle sequencing kit (Perkin-Elmer).

(iii) PCR-RFLP. We took advantage of the occurrence of restriction sites for specific enzymes to perform PCR-restriction fragment length polymorphism (RFLP) as a rapid method of detection of the mutation. The method used to detect the mutations without sequencing involved restric-

tion of the PCR products. Ten microliters of the fragment A amplicon (425 bp) were treated with enzyme *Bsu*II or *Bgl*II (New England Biolabs, Beverly, Mass.). The fragments were incubated for 24 h at 56°C for *Bsu*II and at 37°C for *Bgl*II in order to detect the restriction site occurring when the mutation was A→G at position 2144 or at position 2143, respectively.

Ribosome isolation and erythromycin binding assays. Studies of the binding of radiolabelled antibiotics to ribosomes, isolated from four pairs of strains by the filter binding method, were carried out as described by Goldman et al. (11) and Doucet-Populaire et al. (6) with slight modifications. Briefly, cells were grown on Wilkins Chalgren blood agar, suspended in brucella broth, centrifuged (4000 × g for 15 min), and washed in phosphate-buffered saline and in buffer (10 mM Tris-HCl, 4 mM MgCl₂, 10 mM NH₄Cl, 100 μ M KCl, pH 7.2). Cell lysis was performed by sonication (five cycles of 60 s each with a 60% active cycle) with a Soniprep 150 sonicator (Bioblock Vaaxes, France) at 20 kHz, and ribosome extraction was performed by differential centrifugation (30,000 × g for 30 min at 4°C followed by centrifugation of the supernatant at 100,000 × g for 30 min). The binding of [*N*-methyl-¹⁴C]erythromycin (200 nM) to increasing concentrations of 70 S ribosomes (optical density, 1, 2, 3, and 4) was performed as described by Doucet-Populaire et al. (6).

RESULTS

MIC determination. The isolate obtained from each of the seven patients prior to treatment was susceptible to all antibiotics tested (MIC, 0.03 to 0.125 μ g/liter). The clarithromycin MIC for the second isolate obtained from each patient after an unsuccessful treatment with a regimen including clarithromycin exhibited marked increase (to >2 mg/liter) (Table 1). Cross-resistance to the other macrolides and lincosamides screened by E test and disk diffusion was found. In contrast, the isolates remained susceptible to pristinamycin with one exception (isolate 825). All the strains were susceptible to tetracycline and chloramphenicol.

RAPD fingerprinting. The profiles obtained were similar for each pair of strains but different from one pair to the other (Fig. 1), indicating the persistence of the same strain despite the treatment received.

Detection of mutations. The complete sequence of the peptidyltransferase region of the 23S rRNA gene was determined for each isolate by sequencing 800 nucleotides (fragments A and B). A mutation in domain V of the 23S rRNA was detected in all isolates which were resistant to macrolides and lincosamides but in none of the parent strains which were susceptible to the agents (Table 1). Mutations were found in positions which are homologous to positions 2058 and 2059 of *E. coli*. They were transition mutations (A→G at position 2144 [four cases] and at position 2143 [two cases]) and transversion mutation A→C at position 2143 [one case].



FIG. 1. RAPD patterns (primer 3881) of seven pairs of macrolide-susceptible (S) pre-treatment and macrolide-resistant (R) post-treatment *H. pylori* isolates. Lanes 1, 594S; 2, 677R; 3, 638S; 4, 675R; 5, 646S; 6, 683R; 7, 782R; 9, 803S; 10, 825R; 11, 848S; 12, 1060R; 13, 1054S; 14, 1069R; 15 and 16, CIP 101260; M, DNA marker (*Φ*X 174 *Hae*III).

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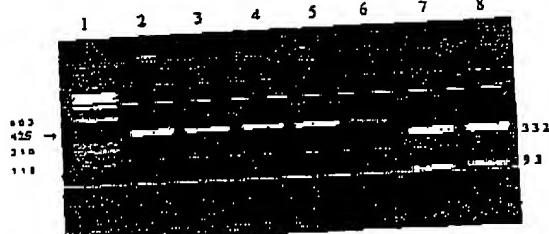


FIG. 2. Restriction profiles of fragment A treated with *Bst*I for three pairs of *H. pylori* strains. Lanes: 1, DNA marker (ΦX174 *Hae*III); 2, amplicon of CIP *H. pylori* strains; 3, 803; 4, 645; 5, 848 (susceptible wild-type strain); 6, 825 (resistant mutant [A→C at position 2143] strain); 7, 685; 8, 1060 (resistant mutant [A→G at position 2143] strain).

It was possible to detect the A→G transition mutations at positions 2144 and 2143 by restriction with *Bsa*I and *Bbs*I, respectively, as expected from the determined sequence (Fig. 2). However, this strategy failed to detect the transversion A→C.

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Binding of [*N*-methyl-¹⁴C]erythromycin to *H. pylori* ribosomes. Figure 3 presents the results obtained for the four pairs of strains tested. There was an increased binding of [*N*-methyl-¹⁴C]erythromycin when the quantity of ribosomes of the susceptible parent strain increased, while virtually no binding was observed with ribosomes from the derived resistant strains.

DISCUSSION

Following the recognition of the important pathogenic role of *H. pylori* infection in the development of gastroduodenal diseases, there has been a continuous search for improved eradication therapy. Clarithromycin emerged as one of the antibiotics of choice because of its low MIC, which is relatively unaffected by lowering the pH, as well as its high concentration in gastric mucosa. In binding experiments the tightest interaction for a macrolide-ribosome complex observed to date was found for the binding of clarithromycin to *H. pylori* ribosomes (10, 11). However, clarithromycin used as a single antibiotic cannot eradicate more than 70% of strains, and resistant strains have been isolated from patients who were not cured.

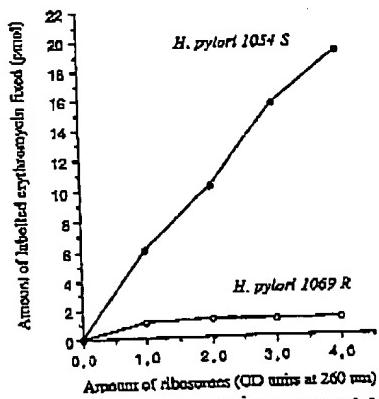
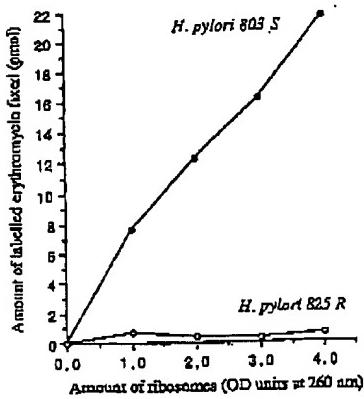
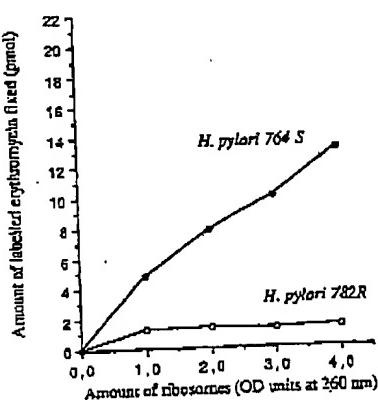
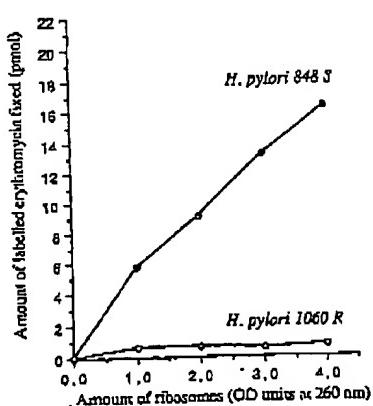


FIG. 3. Binding of [*N*-methyl-¹⁴C]erythromycin (200 nM) to increasing concentrations of 70 S ribosomes (optical densities [OD], 1, 2, 3, 4) purified from four pairs of clarithromycin-susceptible and -resistant *H. pylori* isolates.

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When a second antibiotic was added, the success rate increased to around 90% but resistant strains were still isolated from case failures (17). Furthermore, the actual level of resistance observed (between 1 and 10%, depending on the country) jeopardizes the success of therapy and seems to be related to the extent of consumption of macrolides. It is hypothesized, but not proven, that the use of macrolides to treat respiratory infection leads to resistance in *H. pylori*. Data from France where the consumption of macrolides for treating respiratory tract infection has been at a high level since 1982 have shown a stable resistance frequency in the range of 10% since 1985 (18).

This important problem of clarithromycin resistance led Versalovic et al. (33) to explore the genetic basis of the phenomenon in *H. pylori*. It had been reported that a mutation in the 23S rRNA peptidyltransferase domain was associated with macrolide resistance in *E. coli* (27, 34), *M. avium* (22), *M. intracellulare* (20), and *Mycoplasma pneumoniae* (15). Versalovic et al. studied 12 strains isolated from seven patients in the U.S. and also found that the peptidyltransferase loop of 23S rRNA contained a mutation. They described point mutations at positions 2143 (three cases) and 2144 (four cases). In a recent study performed in The Netherlands (5) five cases of each of these two mutations were described. In our study we confirmed that these mutations are also found in France, a country with a relatively high prevalence of clarithromycin resistance (10%). Furthermore, recent research on rRNA methylases using a conserved-primer PCR assay (2) did not reveal any evidence of *erm*-like genes in *H. pylori* strains (5).

We could also show by sequencing the entire domain of the 23S rRNA that no mutation was present in another site of the domain V loop, for example at positions 2032, 2057, and open-site 2611, sites described for *E. coli* (7, 32). Furthermore we detected a different mutation (24) at position 2143 (A→C instead of A→G), which was also reported recently (29). The MICs of other macrolides and related compounds determined for these strains confirmed that resistance was of concern for all macrolides but that the *H. pylori* strains remained susceptible to pristinamycin, except in the one case. The identity of the strains for a given patient was indicated by similar RAPD profiles before and after treatment including clarithromycin. This result shows that resistance was the result of the selection of variants of the infecting strain rather than infection with a different resistant strain.

In this study, testing of macrolide binding to free *H. pylori* ribosomes from resistant strains was also performed for the first time. Because of the cross-resistance observed between the different macrolides, labelled erythromycin was used in these experiments. The dose response observed, showing the binding of labelled erythromycin to the susceptible parent strains and the absence of binding to the resistant strains, strongly suggests that the lack of binding is the mechanism involved in the occurrence of resistance. We can therefore postulate that this lack of binding is due to a modification in the ribosomal structure following the mutation.

In summary a strong association between point mutations at positions 2143 and 2144 in the 23S rRNA genes, conformational changes in the ribosome at the macrolide binding site, and cross-resistance to macrolides was found. These results suggest that there is a causal relationship. The number of copies of the rRNA operons is relevant to the resistance mechanism. If one copy is present, the resistance is always expressed. Bukanov and Berg (3) and Tomb et al. (31) reported that *H. pylori* had two copies of the rRNA operon, but only two strains were studied. Our results and those of others nevertheless indicate the presence of only one copy because, when

sequencing was performed, only the allelic mutant was found. Nevertheless, Versalovic et al. described a heterozygote resistant strain in one instance. The fraction of resistant ribosomes alone may be sufficient to confer a selectable resistance in *Streptomyces ambofaciens* as well (25).

We think that the possibility of a variable number of copies of the 23S rRNA depending on the strain exists, especially in the light of the important genome variability of *H. pylori*.

Sequencing is obviously the best approach to detect mutations, but it is rather tedious and time consuming even when automated sequencing is used; therefore, we took advantage of the occurrence of new restriction sites on the amplified fragment to detect the mutation by PCR-RFLP. Using enzymes *Bsa*I and *Bbs*I we were able to discriminate between the mutation A→G at positions 2144 and 2143, respectively. This approach could be useful when exploring the epidemiology of macrolide resistance in the future. However, mutation A→C at position 2143 did not produce a restriction site for common enzymes.

The factors that most influence the emergence of drug-resistant strains include inappropriate treatment regimens and lack of compliance of patients. However because of the difficulties in obtaining cultures of *H. pylori* and the delay in obtaining the results, susceptibility testing is rarely carried out before a first treatment or when a second treatment is administered following a treatment failure.

The characterization of resistance mechanisms in *H. pylori* and their easy detection will facilitate the choice of appropriate treatment regimens and ultimately the control of infection.

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